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Similar roles for yeast Dbp2 and *Arabidopsis* RH20 DEAD-box RNA helicases to Ded1 helicase in tombusvirus plus-strand synthesis

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ABSTRACT

Recruited host factors aid replication of plus-strand RNA viruses. In this paper, we show that Dbp2 DEAD-box helicase of yeast, which is a homolog of human p68 DEAD-box helicase, directly affects replication of *Tomato bushy stunt virus* (TBSV). We demonstrate that Dbp2 binds to the 3′-end of the viral minus-stranded RNA and enhances plus-strand synthesis by the viral replicase in a yeast-based cell-free TBSV replication assay. *In vitro* data with wt and an ATPase-deficient Dbp2 mutant indicate that Dbp2 unwinds local secondary structures at the 3′-end of the TBSV (−)RNA. We also show that Dbp2 complements the replication deficiency of TBSV in yeast containing reduced amount of Ded1 DEAD-box helicase, another host factor involved in TBSV replication, suggesting that Dbp2 and Ded1 helicases play redundant roles in TBSV replication. We also show that the orthologous AtRH20 DEAD-box helicase from *Arabidopsis* can increase tombusvirus replication *in vitro* and in yeast.

Introduction

Replication of plus-stranded (+)RNA viruses is a well-orchestrated set of events in infected cells that depends on various viral and host factors. After translation of the mRNA-sense genomic RNA(s), the viral (+)RNA and the viral replication proteins are recruited to the site of viral replication in membranous compartments. This leads to the assembly of the membrane-bound viral replicase complexes (VRCs), the activation of the polymerase function of the viral RNA-dependent RNA polymerase (RdRp), followed by initiation of complementary RNA synthesis on the viral (+)RNA template (den Boon and Ahlquist, 2010; den Boon et al., 2010; Nagy, 2008; Nagy and Pogany, 2012). Replication then switches to (+)-strand synthesis in an asymmetric manner, producing excess amounts of (+)-strand progeny, which is released from replication to enter other viral processes.

Tremendous progress in the last ten years revealed that (+)RNA viruses recruit numerous host proteins to facilitate each step of the replication process (Bartenschlager et al. (2010); Brinton, 2001; den Boon et al., 2010; Nagy, 2008; Nagy and Pogany, 2006, 2012; Nagy et al., 2011; Novoa et al., 2005; Shi and Lai, 2005). One of the largest groups of host factors identified is the RNA-binding proteins (RBPs), such as translation factors, helicases, ribosomal proteins and RNA-modifying enzymes (Li and Nagy, 2011; Nagy, 2008; Ogram and Flanegan, 2011). The co-opted RBPs likely affect several steps in viral RNA replication, including viral (+)RNA recruitment, the assembly of the replicase complex and/or viral RNA synthesis.

Tomato bushy stunt virus (TBSV) is a plant RNA virus with a single genomic RNA, which codes for two replication proteins, p33 and $p92^{pol}$ required for TBSV replicon (rep)RNA replication in yeast (*Saccharomyces cerevisiae*) model host (Panavas and Nagy, 2003; Panaviene et al., 2004). Genome-wide screens and global proteomics approaches with TBSV using yeast identified ~300 host factors, which interact with viral replication proteins or affect TBSV replication. A large fraction of the identified host proteins are RBPs, which might affect viral RNA synthesis (Jiang et al., 2006; Li et al., 2008, 2009; Mendu et al., 2010; Nagy, 2011; Nagy and Pogany, 2010; Panavas et al., 2005; Serva and Nagy, 2006).

The membrane-bound tombusvirus VRC contains p33 and $p92^{pol}$, and the tombusviral (+)repRNA, serving both as a template and as a platform during VRC assembly and activation (Nagy and Pogany, 2008; Panaviene et al., 2005; Pathak et al., 2012; Pogany et al., 2005; Wu et al., 2009). The tombusvirus VRC contains at least seven host proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH, encoded by *TDH2* and *TDH3* in yeast) (Wang and Nagy, 2008), the heat shock protein 70 chaperones (Hsp70, Ssa1/2p in yeast) (Pogany et al., 2008; Serva and Nagy, 2006; Wang et al., 2009b, 2009c), pyruvate decarboxylase (Pdc1p) (Serva and Nagy, 2006), Cdc34p E2 ubiquitin conjugating enzyme (Li et al., 2008), eukaryotic translation

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elongation factor 1A (eEF1A) (Li et al., 2009', 2010), eEF1Bγ (Sasvari et al., 2011), Ded1p DEAD-box helicase (Kovalev et al., 2012) and two temporary resident proteins, Pex19p shuttle protein (Pathak et al., 2008) and the Vps23p adaptor ESCRT protein (Barajas et al., 2009; Barajas and Nagy, 2010; Li et al., 2009). While Hsp70, eEF1A and Vps23p are involved in the assembly of the viral VRC, the functions of host RBPs, such as eEF1A, eEF1Bγ, GAPDH and Ded1p, are to regulate viral RNA synthesis by the VRC (Kovalev et al., 2012; Nagy and Pogany, 2010', 2012; Pogany et al., 2008; Sasvari et al., 2011; Wang and Nagy, 2008; Wang et al., 2009b', 2009c)).

Genome-wide approaches identified several host RNA helicases affecting TBSV replication and the detailed characterization of Ded1p DEAD-box helicase unraveled a major role in (+)-strand synthesis (Kovalev et al., 2012). DEAD-box proteins constitute the largest family of RNA helicases. These RBPs perform RNA duplex unwinding and remodeling of RNA-protein complexes in cells and they are involved in all aspects of cellular metabolism (Cordin et al., 2006; Linder, 2008; Linder and Lasko, 2006).

In this paper, we have characterized the role of an ATP-dependent RNA helicase of the large DEAD-box protein family, namely Dbp2p in TBSV replication. Dbp2p is an essential, mostly cytosolic yeast protein involved in nonsense-mediated mRNA decay and rRNA processing (Bond et al., 2001). Dbp2p, one of 20 yeast DEAD-box proteins, is the homolog of the human p68 protein (Barta and Iggo, 1995). Dbp2p also affects protein translation and translation fidelity (Bond et al., 2001). We have previously found that purified Dbp2p bound to the TBSV replicon RNA *in vitro* and two-step affinity purification of Dbp2p from yeast also resulted in co-purification of the TBSV repRNA (Li et al., 2009). Over-expression of Dbp2p in yeast led to enhanced level of TBSV RNA accumulation, suggesting that Dbp2p might play a role in TBSV replication.

To define the possible role of Dbp2p in TBSV replication, here we used a yeast-based cell-free TBSV replication assay and recombinant Dbp2p. We show that Dbp2p plays a role in enhancing plus-strand (+)RNA synthesis by the tombusviral replicase. We also find that Dbp2p binds to the 3′-end of the viral minus-stranded (−)RNA. Based on data presented, we propose that Dbp2p unwinds the 3′-end of the TBSV (−)RNA, and facilitates initiation of (+)-strand synthesis. Interestingly, we find that Dbp2p plays a redundant role with Ded1p, another DEAD-box helicase, in enhancing (+)-strand synthesis. We also show that the *Arabidopsis* homolog, AtRH20 DEAD-box helicase faciltates tombusvirus replication *in vitro* and in yeast. Altogether, we propose that a group of DEAD-box helicases facilitate replication of tombusviruses, which do not code for their own RNA helicases.

Results

Dbp2p helicase and the homologous AtRH20 DEAD-box helicase bind to the 3′ end of the TBSV (−)RNA in vitro

We have previously purified Dbp2p–TBSV replicon (rep)RNA complex from yeast cells, suggesting the Dbp2p might be involved in TBSV RNA replication (Li et al., 2009). To identify the region(s) of the TBSV RNA bound by the recombinant Dbp2p, we performed electrophoresis mobility shift assay (EMSA) with purified components. We found that RIII(+) and RIV(+), representing mostly the 3′ UTR in the TBSV genomic (g)RNA (Fig. 1A), were bound by the recombinant Dbp2p (Fig. 1B, lanes 8–9 and 11–12) more efficiently than RI(+) (representing the 5′UTR) and RII(+) (representing an internal coding region within the p92 ORF, Fig. 1A). Interestingly, the binding of Dbp2p to RIII(+) and RIV(+) was not outcompeted by the unlabeled DI-72 (+)repRNA (Fig. 1B, lanes 9 and 12).

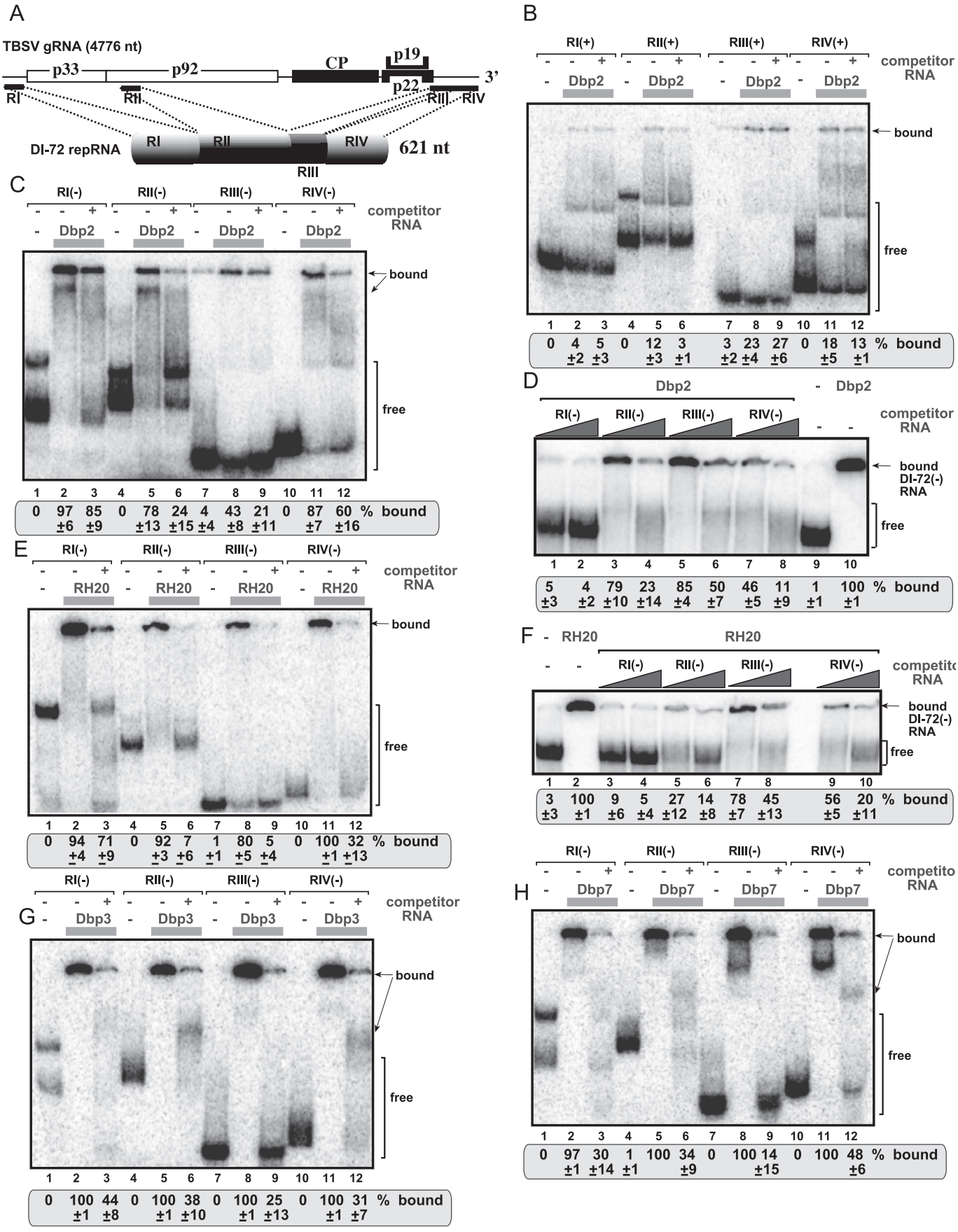
Additional EMSA experiments with the four regions in DI-72 (−)repRNA (Fig. 1C) revealed that Dbp2p bound to each region, but binding to the RI(−) sequence was the most efficient (Fig. 1C, lane 2). Also, using an unlabeled TBSV (−)repRNA as a competitor RNA in the EMSA experiment revealed that the ^{32}P-labeled RI(−) was the least efficiently outcompeted (Fig. 1C, lane 3). Moreover, RI(−) was the most efficient competitor among the four regions when they were used as unlabeled RNA probes in the EMSA experiment based on the ^{32}P-labeled DI-72 (−)RNA (Fig. 1D, lanes 1–2). Altogether, comparison of ^{32}P-labeled TBSV (+) and (−)repRNA regions in binding to purified recombinant Dbp2p revealed that RI(−) RNA bound to Dbp2p the most efficiently *in vitro* (Fig. 1B–D). This could be important for the function of Dbp2p in TBSV replication since RI(−) represents the 3′ end of (−)repRNA and contains important *cis*-acting elements, such as the promoter and a short enhancer sequence for (+)RNA synthesis (Panavas et al., 2003', 2002a).

The closest homolog of Dbp2p in *Arabidopsis thaliana* model plant is the cytosolic AtRH20 DEAD-box helicase (Fig. 2A). AtRH20 has been shown to interact with the tombusvirus p33 and stimulated RNA synthesis by the purified tombusvirus replicase *in vitro* (Kovalev et al., 2012). To compare the characteristics of AtRH20 with the yeast Dbp2p, we also expressed and purified AtRH20 from *Escherichia coli* and tested its ability to bind to TBSV repRNA. Using EMSA, we found that AtRH20 bound to RI(−) more efficiently (Fig. 1E, lane 2) than to the other regions in DI-72 (−)repRNA. In addition, RI(−) was the most efficient competitor for AtRH20 among the four regions in DI-72 (−)repRNA in the EMSA experiment (Fig. 1F, lanes 3–4). Thus, AtRH20 shows remarkable similarity to the yeast Dbp2p in viral RNA binding *in vitro*.

Another yeast DEAD-box helicase, Ded1p, which has high sequence similarity with the yeast Dbp2p (Fig. 2B), AtRH20 and human DDX3x (not shown), has also been shown to bind to RI(−) more efficiently than to other regions of the TBSV repRNA (Kovalev et al., 2012). This suggests that Ded1p and Dbp2p helicases might have similar functions in TBSV replication. To test if other yeast DEAD-box helicases might show similar binding specificity toward TBSV (−)repRNA, we selected Dbp3p, which has been identified in a previous screen, showing Dbp3p could bind to the TBSV p92^{pol} replication protein *in vitro* (Li et al., 2008). Dbp3p is a putative ATP-dependent RNA helicase of the DEAD-box family involved in ribosomal biogenesis (Weaver et al., 1997). In addition, we tested Dbp7p DEAD-box helicase, which is also involved in ribosome biogenesis (Bernstein et al., 2006; Daugeron and Linder, 1998), and over-expression of Dbp7p decreases TBSV repRNA accumulation in yeast (not shown). The EMSA assay with purified recombinant Dbp3p and Dbp7p revealed that these helicase proteins bind to each region in DI-72(−) RNA (Fig. 1G and H). Interestingly, the binding of Dbp3p and Dbp7p to TBSV (−)RNA regions was readily outcompeted by DI-72(−) RNA (Fig. 1G and H), demonstrating that the TBSV RNA-binding characteristic of other yeast DEAD-box helicases is different from that of Dbp2p and Ded1p.

Dbp2p and AtRH20 promote TBSV (+)RNA synthesis by the purified tombusvirus replicase

Binding of Dbp2p to the RI(−) sequence suggests that Dbp2p could be involved in regulation of (+)-strand synthesis, which is controlled by RI(−) (Panavas et al., 2003 , 2002a). To test the direct effect of Dbp2p on (+)RNA synthesis, we utilized a detergent-solubilized and affinity-purified tombusvirus replicase preparation (Fig. 3A). This purified replicase can only produce complementary RNA on added TBSV templates, but cannot perform a complete cycle of RNA synthesis (Panaviene et al., 2005', 2004). For this assay, we used purified tombusvirus replicase


A
TBSV gRNA (4776 nt)
p33
p92
CP
p19
p22
3'
RI
RII
RIII
RIV
DI-72 repRNA
621 nt
B
RI(+)
RII(+)
RIII(+)
RIV(+)
competitor RNA
Dbp2
bound
free
1 2 3 4 5 6 7 8 9 10 11 12
0 4 5 0 12 3 3 23 27 0 18 13 % bound
±2 ±3 ±3 ±1 ±2 ±4 ±6 ±5 ±1
C
RI(-)
RII(-)
RIII(-)
RIV(-)
competitor RNA
Dbp2
bound
free
1 2 3 4 5 6 7 8 9 10 11 12
0 97 85 0 78 24 4 43 21 0 87 60 % bound
±6 ±9 ±13 ±15 ±4 ±8 ±11 ±7 ±16
D
Dbp2
- Dbp2
RI(-)
RII(-)
RIII(-)
RIV(-)
competitor RNA
bound DI-72(-) RNA
free
1 2 3 4 5 6 7 8 9 10
5 4 79 23 85 50 46 11 1 100 % bound
±3 ±2 ±10 ±14 ±4 ±7 ±5 ±9 ±1 ±1
E
RI(-)
RII(-)
RIII(-)
RIV(-)
competitor RNA
RH20
bound
free
1 2 3 4 5 6 7 8 9 10 11 12
0 94 71 0 92 7 1 80 5 0 100 32 % bound
±4 ±9 ±3 ±6 ±1 ±5 ±4 ±1 ±13
F
- RH20
RH20
RI(-)
RII(-)
RIII(-)
RIV(-)
competitor RNA
bound DI-72(-) RNA
free
1 2 3 4 5 6 7 8 9 10
3 100 9 5 27 14 78 45 56 20 % bound
±3 ±1 ±6 ±4 ±12 ±8 ±7 ±13 ±5 ±11
G
RI(-)
RII(-)
RIII(-)
RIV(-)
competitor RNA
Dbp3
bound
free
1 2 3 4 5 6 7 8 9 10 11 12
0 100 44 0 100 38 0 100 25 0 100 31 % bound
±1 ±8 ±1 ±10 ±1 ±13 ±1 ±7
H
RI(-)
RII(-)
RIII(-)
RIV(-)
competitor RNA
Dbp7
bound
free
1 2 3 4 5 6 7 8 9 10 11 12
0 97 30 1 100 34 0 100 14 0 100 48 % bound
±1 ±14 ±1 ±9 ±15 ±6

preparations obtained from yeast expressing low level of Ded1p to reduce the amount of co-purified DEAD-box helicase in the replicase preparation (Kovalev et al., 2012) (see below).

The *in vitro* tombusvirus replicase assay revealed that the addition of purified recombinant Dbp2p to the purified tombusvirus replicase using the (−)repRNA as a template led to increased (+)-strand synthesis by ~2-fold (Fig. 3B, lane 2 *versus* 3). Interestingly, Dbp2p stimulated the production of the full-length (+)-strand RNA product, which is the result of *de novo* initiation. On the contrary, the amount of internal initiation products (also due to *de novo* initiation, marked as "ii" in Fig. 3B) and a 3′-terminal extension product [3′TEX; due to self-priming by the 3′ end of the template (Cheng et al., 2002; Nagy and Pogany, 2000; Panavas et al., 2002b)] decreased in the presence of added recombinant Dbp2p. These data indicate that Dbp2p specifically facilitates the 3′ terminal *de novo* initiation on the (−)RNA template by the tombusvirus replicase.

Comparison between Ded1p and Dbp2p helicases revealed that Ded1p is more efficient in facilitating TBSV (+)RNA synthesis in tombusvirus replicase assay (compare lanes 3 and 5 in Fig. 3C). Also, the *Arabidopsis* DEAD-box helicase, AtRH20, which is homologous with both Dbp2p (Fig. 2) and Ded1p (Kovalev et al., 2012), promoted TBSV (+)RNA synthesis in tombusvirus replicase assay as efficiently as Dbp2p (Fig. 3C, lanes 7 *versus* 5). This suggests that AtRH20 could have similar function to Dbp2p in TBSV replication.

To examine if the ATPase activity of Dbp2p is required for the above stimulatory effect on TBSV (+)RNA synthesis, we used an ATPase inactive mutant (F366L, Fig. 3B, lanes 4–6) (Banroques et al., 2008) in the purified tombusvirus replicase assay. These experiments revealed that the F366L mutant of Dbp2p inhibited (+)-strand synthesis, especially when used in high concentration (Fig. 3C, lane 6). Similarly, the ATPase-inactive mutant of Ded1p did not stimulate RNA synthesis in the purified tombusvirus replicase assay (D1 mutant, Fig. 3C, lane 4) (Kovalev et al., 2012). Thus, these data suggest that, similar to Ded1p, the ATPase activity of Dbp2p is required for the stimulation of TBSV (+)RNA synthesis *in vitro*.

To test if Dbp2p could facilitate (+)RNA synthesis by another viral replicase, we selected the Flock house virus (FHV) replicase, which we have developed based on affinity-purified FHV replicase preparation that can be programmed with exogenously added RNAs (Kovalev et al., 2012). The *in vitro* FHV replicase assay revealed that the purified recombinant Dbp2p enhanced (+)-strand RNA synthesis on the (−)-stranded FHV template by ~2.5-fold (Fig. 3D, lanes 2 *versus* 3). The F366L ATPase inactive mutant of Dbp2p slightly decreased (+)RNA synthesis by the FHV replicase (Fig. 3D, lane 1). Thus we conclude that the ATPase activity of Dbp2p is important for the stimulatory function on (+)RNA synthesis by either the tombusvirus or FHV replicases *in vitro*. < empty >

Dbp2p enhances (+)-strand RNA initiation by the tombusvirus replicase on a partial RNA/DNA duplex

To test if Dbp2p could function by remodeling RNA structure to facilitate RNA synthesis by the viral replicase, we used a partial DNA/RNA duplex, which is known to hinder RdRp-driven RNA synthesis in the tombusvirus and FHV replicase assays (Kovalev et al., 2012; Panavas et al., 2006; Stork et al., 2011). The short DNA oligo hybridized to the promoter region of (−)RNA (Fig. 4A) can inhibit (+)RNA synthesis by the tombusvirus replicase *in vitro* by "masking" the sequence of the plus-strand initiation promoter (Panavas et al., 2002a, 2002b; Stork et al., 2011). We chose a partial duplex for this assay, since Dbp2p and other DEAD-box helicases are not processive enzymes and can only unwind short duplexes (Linder and Lasko, 2006). We found that the purified Dbp2p enhanced (+)RNA synthesis by ~50% in the tombusvirus replicase assay (Fig. 4B, lane 2) and by ~150% in the FHV replicase assay (Fig. 4C, lane 2) containing the partial DNA/RNA duplex. The F366L ATPase deficient mutant of Dbp2p could not stimulate RNA synthesis on the partial DNA/RNA duplex by tombusvirus or FHV replicases (Fig. 4B and C, lane 3). Thus, the ATPase/helicase function of Dbp2p is necessary for the stimulatory effect on viral (+)RNA synthesis on a short DNA/RNA duplex. These data suggest that RNA unwinding/remodeling function of Dbp2p is likely important for TBSV and possibly FHV RNA replication.

To demonstrate that the purified Dbp2p has nucleic-acid unwinding activity, we used the above partial DNA/RNA duplex in a strand separation assay (Stork et al., 2011). This *in vitro* assay revealed that the wt Dbp2p DEAD-box RNA helicase was capable of complete separation of the partial ssDNA/ssRNA duplex (Fig. 4D, lanes 8–10). As expected, ATP stimulated the unwinding activity of Dbp2p (Fig. 4D, lane 10 *versus* lane 7). The F366L ATPase deficient mutant of Dbp2p could not separate the partial RNA/DNA duplex under the same conditions (Fig. 4D, lanes 1–3). These data are consistent with the model that the ATP-driven unwinding activity of Dbp2p could facilitate initiation of RNA synthesis by the tombusvirus replicase *in vitro*.

Recombinant Dbp2p helicase promotes TBSV repRNA replication in a yeast cell-free extract

The above features of Dbp2p including binding to RI(−) and stimulating (+)-strand synthesis in the tombusvirus replicase assay *in vitro*, are similar to those we described for Ded1p DEAD-box helicase (Kovalev et al., 2012). These similarities between Dbp2p and Ded1p suggest that these helicases might play complementary roles in TBSV replication. We tested this possibility using whole cell extracts (CFE) prepared from yeast to support cell-free TBSV replication (Fig. 5A). As we have demonstrated previously, TBSV (+)RNA can perform one full cycle of replication in the CFE-based replication assay in the presence of added purified recombinant p33 and p92^{pol} replication proteins (Pogany and Nagy, 2008; Pogany et al., 2008). To address if Dbp2p could stimulate TBSV replication, we added purified recombinant Dbp2p to yeast CFE containing reduced level of Ded1p (Fig. 5B). We observed ~60% increase in TBSV repRNA replication in the presence of recombinant Dbp2p (Fig. 5B, lanes 5–6 *versus* 3–4), while F366L mutant did not increase TBSV replication (based

Fig. 1. Dbp2p and the *Arabidopsis* homolog AtRH20 DEAD-box helicases bind to the 3′ end of the TBSV (−)RNA. (A) Schematic representation of the four regions (RI-to-RIV) in DI-72 repRNA used in the binding assay. (B) *In vitro* EMSA binding assay with purified Dbp2p using ^{32}P-labeled ssRNA templates. The assay contained one of the four regions of the DI-72 (+)repRNA (~0.1 pmol), plus 0.6 μg (lanes 2, 3, 5, 6, 8, 9, 11 and 12) of purified recombinant Dbp2p, as shown. Unlabeled DI-72 (+)RNA was used in 0.3 μg amount (lanes 3, 6, 9 and 12) as competitor. The free or Dbp2p-bound ssRNA was separated on nondenaturing 5% acrylamide gels. (C) RNA gel shift analysis shows that Dbp2p binds the most efficiently to RI(−). ^{32}P-labeled template RNA (~0.1 pmol) represented one of the four (−)-stranded regions, as shown. See further details in Panel B. Each experiment was repeated at least two times. (D) Competition assay based on RNA gel shift analysis shows that Dbp2p binds the most efficiently to RI(−). ^{32}P-labeled DI-72 (−)repRNA template (~0.1 pmol) and unlabeled competitor RNAs (3 and 6 pmol) representing one of the four regions (see panel A) were used in the competition assay. The Dbp2p–^{32}P-labeled ssRNA complex was visualized on nondenaturing 5% acrylamide gels. (E) *In vitro* EMSA binding assay with purified AtRH20 (the *Arabidopsis* homolog of Dbp2p) DEAD-box helicase using ^{32}P-labeled (−)repRNA as shown. See further details in Panel C. (F) RNA gel shift analysis shows that AtRH20 binds the most efficiently to RI(−). ^{32}P-labeled DI-72 (−)repRNA template (~0.1 pmol) and unlabeled competitor RNAs (3 and 6 pmol) representing one of the four regions (see panel A) were used in the competition assay. See further details in Panel D. (G)–(H) *In vitro* EMSA binding assay with purified Dbp3p or Dbp7p DEAD-box helicases using ^{32}P-labeled (−)repRNA as shown. See further details in Panel C.

A

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Dbp2   66    ELPKLPTFEKNFYVEHESVRDRSDSEIAQFRKENEMTISGHDIPKPITTFDEAGFPDYVL  125
             +L  L  FEKNFYVE  +V   +D+E+ ++RK  E+T+ G DIPKP+ +F + GFPDYVL
AtRH20 52    DLDGLTPFEKNFYVESPAVAAMTDTEVEEYRKLREITVEGKDIPKPVKSFRDVGFPDYVL  111

Dbp2   126   NEVKAEGFDKPTGIQCQGWPMALSGRDMVGIAATGSGKTLSYCLPGIVHINAQPLLAPGD  185
              EVK  GF +PT IQ QGWPMA+ GRD++GIA TGSGKTLSY LP IVH+NAQP+LA GD
AtRH20 112   EEVKKAGFTEPTPIQSQGWPMAMKGRDLIGIAETGSGKTLSYLLPAIVHVNAQPMLAHGD  171

Dbp2   186   GPIVLVLAPTRELAVQIQTECSKFGHSSRIRNTCVYGGVPKSQQIRDLSRGSEIVIATPG  245
             GPIVLVLAPTRELAVQIQ E SKFG SS+I+ TC+YGGVPK  Q+RDL +G EIVIATPG
AtRH20 172   GPIVLVLAPTRELAVQIQQEASKFGSSSKIKTTCIYGGVPKGPQVRDLQKGVEIVIATPG  231

Dbp2   246   RLIDMLEIGKTNLKRVTYLVLDEADRMLDMGFEPQIRKIVDQIRPDRQTLMWSATWPKEV  305
             RLIDM+E   TNL+RVTYLVLDEADRMLDMGF+PQIRKIV  IRPDRQTL WSATWPKEV
AtRH20 232   RLIDMMESNNTNLRRVTYLVLDEADRMLDMGFDPQIRKIVSHIRPDRQTLYWSATWPKEV  291

Dbp2   306   KQLAADYLNDPIQVQVGSLELSASHNITQIVEVVSDFEKRDRLNKYLETASQDNEYKTLI  365
             +QL+  +L +P +V +GS +L A+  I QIV+V+S+ +K ++L K LE     +  + L+
AtRH20 292   EQLSKKFLYNPYKVIIGSSDLKANRAIRQIVDVISESQKYNKLVKLLEDIMDGS--RILV  349

Dbp2   366   FASTKRMCDDITKYLREDGWPALAIHGDKDQRERDWVLQEFRNGRSPIMVATDVAARGID  425
             F  TK+ CD IT+ LR DGWPAL+IHGDK Q ERDWVL EFR+G+SPIM ATDVAARG+D
AtRH20 350   FLDTKKGCDQITRQLRMDGWPALSIHGDKSQAERDWVLSEFRSGKSPIMTATDVAARGLD  409

Dbp2   426   VKGINYVINYDMPGNIEDYVHRIGRTGRAGATGTAISFFTEQNKGLGAKLISIMREANQN  485
             VK + YVINYD PG++EDYVHRIGRTGRAGA GTA +FFT  N     +L +I++EA Q
AtRH20 410   VKDVKYVINYDFPGSLEDYVHRIGRTGRAGAKGTAYTFFTVANARFAKELTNILQEAGQK  469

Dbp2   486   IPPEL  490
             + PEL
AtRH20 470   VSPEL  474
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B

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Dbp2   104   SGHDIPKPITTFDEAGFPDYVLNEVKAEGFDKPTGIQCQGWPMALSGRDMVGIAATGSGK 163
             SG D+P+PIT F        +L  +K   F KPT +Q    P+  +GRD++  A TGSGK
Ded1   133   SGKDVPEPITEFTSPPLDGLLLENIKLARFTKPTPVQKYSVPIVANGRDLMACAQTGSGK 192

Dbp2   164   TLSYCLPGIVH-INAQPLLAP-GDG--------PIVLVLAPTRELAVQIQTECSKFGHSS 213
             T  +  P +       P   P   G        P  +++APTRELA QI  E  KF + S
Ded1   193   TGGFLFPVLSESFKTGPSPQPESQGSFYQRKAYPTAVIMAPTRELATQIFDEAKKFTYRS 252

Dbp2   214   RIRNTCVYGGVPKSQQIRDLSRGSEIVIATPGRLIDMLEIGKTNLKRVTYLVLDEADRML 273
             ++   VYGG P   Q+R++ RG ++++ATPGRL D+LE GK +L  V YLVLDEADRML
Ded1   253   WVKACVVYGGSPIGNQLREIERGCDLLVATPGRLNDLLERGKISLANVKYLVLDEADRML 312

Dbp2   274   DMGFEPQIRKIVDQ--IRP--DRQTLMWSATWPKEVKQLAADYLNDPIQVQVGSLELSAS 329
             DMGFEPQIR IV+   + P  +RQTLM+SAT+P +++ LA D+L+D I + VG +  S S
Ded1   313   DMGFEPQIRHIVEDCDMTPVGERQTLMFSATFPADIQHLARDFLSDYIFLSVGRVG-STS 371

Dbp2   330   HNITQIVEVVSDFEKRDRLNKYLETASQDNEYKTLIFASTKRMCDDITKYLREDGWPALA 389
              NITQ V  V   E +D+ +  L+  S   +  TLIF  TKRM D +T +L    + A A
Ded1   372   ENITQKVLYV---ENQDKKSALLDLLSASTDGLTLIFVETKRMADQLTDFLIMQNFRATA 428

Dbp2   390   IHGDKDQRERDWVLQEFRNGRSPIMVATDVAARGIDVKGINYVINYDMPGNIEDYVHRIG 449
             IHGD+ Q ER+  L  FR+G + ++VAT VAARG+D+  + +VINYD+P +++DYVHRIG
Ded1   429   IHGDRTQSERERALAAFRSGAATLLVATAVAARGLDIPNVTHVINYDLPSDVDDYVHRIG 488

Dbp2   450   RTGRAGATGTAISFFTEQNKGLGAKLISIMREANQNIP 487
             RTGRAG TGTA +FF  +N  +   L  I+ EANQ +P
Ded1   489   RTGRAGNTGLATAFFNSENSNIVKGLHEILTEANQEVP 526
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Fig. 2. Comparison of the amino acid sequence of (A) the yeast Dbp2p (top) and the *Arabidopsis* AtRH20 DEAD-box helicases and (B) the yeast Dbp2p (top) and Ded1p helicases.

on total single-stranded repRNA level) when compared with the MBP control (Fig. 5B, lane 7–8 *versus* 3). Interestingly, similar to the *in vitro* TBSV replicase assay (Fig. 3), Dbp2p was also less efficient in stimulation of TBSV replication in the CFE-based assay in comparison with Ded1p helicase (compare lanes 5–6 and 1–2, Fig. 5B).

The CFE-based TBSV replication assay also revealed that the amount of double-stranded RNA, which correlates with (−)-strand levels (Pogany et al., 2008), increased only by ~15% in the presence of Dbp2p (Fig. 5C, lanes 4 *versus* 1 and lanes 6 *versus* 8). This finding suggests that (−)-strand RNA synthesis does not contribute (or contribute only a little bit) to the increase in TBSV (+)repRNA levels when recombinant Dbp2p was present in the CFE-based replication assay. Therefore, we suggest that the added recombinant Dbp2p can complement Ded1p in the CFE assay (containing reduced Ded1p level) by selectively increasing TBSV (+)-strand RNA synthesis *in vitro*. This activity requires the ATPase/helicase function of Dbp2p as shown by the lack of enhancement of TBSV RNA replication in the CFE-based replication assay in the presence of ATPase defective F366L mutant of Dbp2p (Fig. 5C, lane 5).

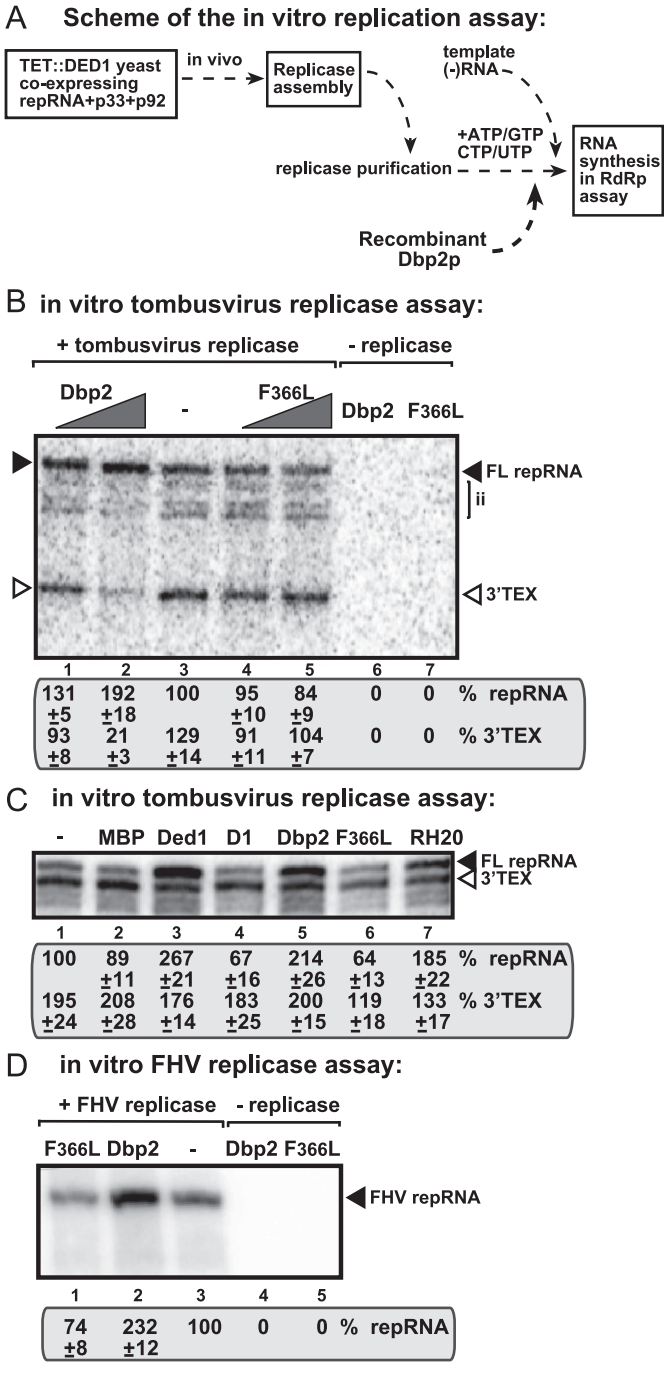

Fig. 3. Dbp2p promotes plus-strand synthesis by the affinity-purified tombusvirus and FHV replicases. (A) Scheme of the tombusvirus replicase assay. Yeast (with depleted Ded1p to decrease the co-purified DEAD-box helicase) co-expressing p33 and $p92^{pol}$ replication proteins and DI-72 (+)repRNA were used to affinity-purify the RNA-free tombusvirus replicase. The *in vitro* assays contained the DI-72 (−)repRNA, the affinity-purified recombinant Dbp2p, the ATPase deficient mutant (called dbp2-F366L) in addition to ATP/CTP/GTP and ^{32}P-UTP. (B) Representative denaturing gel of ^{32}P-labeled RNA products synthesized by the purified tombusvirus replicase *in vitro* in the presence of 0.5 or 1.0 μg of purified recombinant Dbp2p or dbp2-F366L mutant is shown. The level of complementary RNA synthesis producing "repRNA" (marked as "FL", the full-length product, made via *de novo* initiation from the 3′-terminal promoter) in each sample was compared to that of the replicase activity obtained in the absence of added recombinant protein (lane 3). Note that this replicase preparation also synthesizes internal initiation products ("ii") and 3′-terminal extension products ("3′TEX"). The amounts of 3′TEX products were compared to the FL repRNA product (chosen as 100%, lane 3) without normalization for ^{32}P-UTP that was incorporated into the products. Note that the 3′TEX products migrate aberrantly under these conditions due to the very stable hairpin-like structure. Each experiment was repeated three times. (C) Comparison of the stimulatory activities of various DEAD-box helicases on the purified tombusvirus replicase *in vitro*. Representative denaturing gel of ^{32}P-labeled RNA products synthesized in the presence of 1.2 μg of purified recombinant proteins is shown. Note that, after the *in vitro* tombusvirus replicase assay, the samples were treated with RNase One, which cleaves the single-stranded loop region of the hairpin-like structure in the 3′TEX products, resulting in slower migration under the conditions used. While the stimulatory effect of Dbp2p and Ded1p on full-length repRNA was highly repeatable, the inhibitory effect of these recombinant helicases on the 3′TEX product varied from batch-to-batch. See further details in Panel B. (D) Representative denaturing gel of ^{32}P-labeled RNA products synthesized by the purified FHV replicase *in vitro* in the presence of 1.0 μg of purified recombinant Dbp2p or dbp2-F366L mutant is shown. Note that the FHV DI-634 (−)repRNA was used as a template in the FHV replicase assay.

Dbp2p does not facilitate the assembly of the tombusvirus VRC in yeast cell-free extract

To test if viral RNA recruitment into replication or VRC assembly is affected by Dbp2p, we performed a two-step replication assay based on yeast CFE. In this assay, the first step includes viral RNA recruitment to the membrane, followed by the assembly of the replicase complex on the endogenous membranes present in the CFE in the presence of the viral (+)repRNA, the p33/p92 replication proteins and ATP/GTP (Pogany et al., 2008) (Fig. 6A, step 1). Under these conditions, the viral replicase assembles and becomes partially RNase and protease insensitive, but the VRC cannot initiate minus-strand synthesis due to the absence of CTP/UTP (Pogany et al., 2008). Then, all the proteins and molecules not bound to the membrane are removed by centrifugation and washing the membranes to prevent additional VRC assembly during the second step. The second step includes the addition of ATP/CTP/GTP/UTP to allow RNA synthesis.

When we added the purified Dbp2p to the CFE during the first step (Fig. 6A, step 1), TBSV repRNA replication was not increased (Fig. 6B, lane 4 *versus* 3). This suggests that Dbp2p did not facilitate the recruitment of the repRNA into replication or the assembly of the replicase complex. It is also likely that Dbp2p was lost during the centrifugation/washing step in this assay due to its poor association with the membrane.

When we exclusively added Dbp2p during the second step of the CFE assay, then we observed a ~70% increase in TBSV repRNA replication (Fig. 6B, lane 14 *versus* 13), similar to the stimulatory effect of Dbp2p during standard CFE replication assay (Fig. 5B). As expected, F366L mutant of Dbp2p with deficient ATPase activity could not stimulate TBSV RNA synthesis in this assay (Fig. 6B, lane 15). Comparison of the effect of Dbp2p *versus* Ded1p revealed that both helicases stimulated TBSV repRNA replication when added during the second step (*i.e.*, RNA synthesis steps) (Fig. 6B, lanes 11 and 14). These data further support that Dbp2p has a comparable role to Ded1p helicase during TBSV repRNA replication *in vitro*. This behavior of Dbp2p is unlike Hsp70 and

eEF1A host factors, which facilitated TBSV replication when added during the first step of the assay (Li et al., 2010; Pogany et al., 2008). Altogether, these findings suggest that Dbp2p has a direct stimulatory function on (+)-strand synthesis during TBSV RNA synthesis, and Dbp2p might have redundant function with Ded1p helicase, while Dbp2p is unlikely to affect viral RNA recruitment for replication or VRC assembly.

Interaction of Dbp2p with p33 replication co-factor is facilitated by the viral RNA

To test if Dbp2p can interact with the TBSV p33 replication protein, we used the membrane-based split-ubiquitin assay (Barajas and Nagy, 2010). We found that Dbp2p interacted poorly with p33 protein in yeast (Fig. 7A). Also, pull-down experiments with MBP-p33 and MBP-p92 showed no specific binding to Dbp2p *in vitro* (not shown). Thus, Dbp2p does not seem to directly interact with the TBSV replication proteins *in vitro*. This is different from Ded1p, which bound efficiently to the TBSV replication proteins (Kovalev et al., 2012). The binding of AtRH20 to p33 in the split-ubiquitin assay was detectable (Fig. 7A), suggesting that both Ded1p and AtRH20 could directly interact with the tombusvirus replication protein.

To examine if the viral RNA might facilitate the interaction between Dbp2p and p33, we modified the split-ubiquitin assay by launching TBSV repRNA replication in the appropriate yeast strains. Interestingly, the modified assay showed the possible interaction between Dbp2p and p33 only when the repRNA replication occurred in the test yeast strain (right panel in Fig. 7B). It is possible that the viral repRNA serves as a bridge by interacting with both Dbp2p and p33, bringing the protein into proximity and/or stabilizing the interaction between the proteins.

Expression of Dbp2p and AtRH20 helicases stimulates TBSV repRNA accumulation in Ded1p depleted yeast

Dbp2p is essential for yeast growth, thus we could not test TBSV replication in the absence of Dbp2p. However, over-expression of Dbp2p in wt yeast led to 36% more efficient TBSV repRNA accumulation (Li et al., 2009), albeit we only measured 23% increase in TBSV repRNA accumulation in yeast over-expressing Dbp2p in this work (Fig. 8A, lanes 5–8). However, the increase is significant since over-expression of many host proteins generally leads to ~10–25% decrease in TBSV repRNA accumulation (Li et al., 2008', 2009). The increase in TBSV replication driven by Dbp2p over-expression was less than the 41% increase seen with over-expression of Ded1p helicase in yeast (not shown). Interestingly, over-expression of the F366L mutant of Dbp2p decreased TBSV repRNA replication by ~40% (Fig. 8A, lanes 9–12), suggesting that F366L acts as a dominant-negative mutant. Also, over-expression of the homologous AtRH20 helicase led to 46% increase in TBSV replication in yeast (Fig. 8A, lanes 13–16). The amounts of p33 and p92^{pol} produced in yeast over-expressing Dbp2 or F366L mutant did not change when compared to the control samples (Fig. 8A).

To test if Dbp2p could complement the reduced TBSV repRNA replication level in yeast with down-regulated Ded1p, we over-

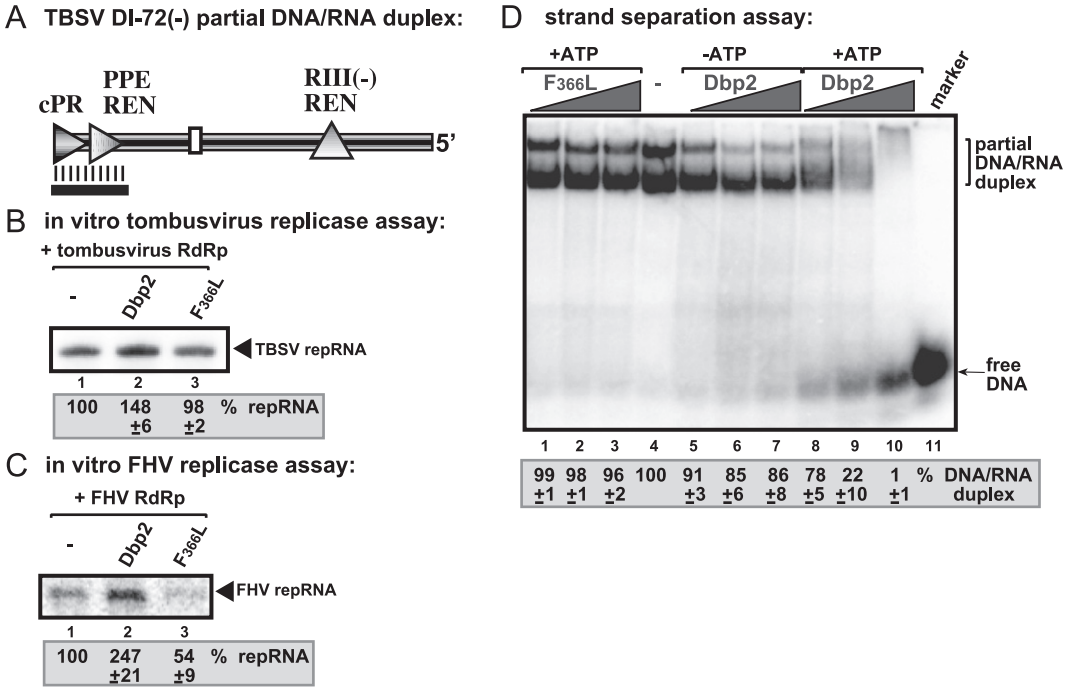

Fig. 4. Dbp2p facilitates the RNA synthesis by the tombusvirus and FHV replicases on short partial DNA/RNA duplex. (A) Schematic representation of the DNA/RNA duplex used in the replicase assays. The unlabeled template consists of DI-72 (−)repRNA and a complementary DNA oligo, which anneals to the 3′ end DI-72 (−)repRNA and forms a 21 nt duplex as shown. The *cis*-acting sequences shown are involved in (+)-strand synthesis: including cPR, which is an 11 nt long sequence used as a (+)-strand initiation promoter; PPE REN is a promoter proximal replication enhancer; and RIII(−)REN is a strong replication enhancer within RIII(−) sequence. (B) Representative denaturing gel of ^{32}P-labeled RNA products synthesized by the purified tombusvirus replicase *in vitro* in the presence of 1.0 μg of purified recombinant Dbp2p or dbp2-F366L mutant. The level of complementary RNA synthesis using the DNA/RNA duplex (panel A) in each sample was compared to that of the replicase activity obtained in the absence of added recombinant protein (lane 1). See further details in Fig. 3. (C) Representative denaturing gel of ^{32}P-labeled RNA products synthesized by the purified FHV replicase *in vitro* in the presence of 1.0 μg of purified recombinant Dbp2p or dbp2-F366L mutant on the DNA/RNA duplex (panel A). Each experiment was repeated three times. (D) Strand separation assay with a partial DNA/RNA duplex shows the unwinding activity of Dbp2p in the presence of ATP. The ^{32}P-labeled DNA oligo was annealed to DI-72 (−)RNA forming a 21 nt duplex as shown in Panel A. The nondenaturing PAGE analysis show the annealed DNA/RNA duplex and the released (free oligo) DNA in the presence of purified recombinant Dbp2p (0.4, 0.8, 1.2 μg) (+ATP or no ATP) and the dbp2-F366L mutant. Quantification of the DNA/RNA duplex is done with a Phosphorimager.

expressed Dbp2p in TET:DED1 yeast, in which Ded1 mRNA level was down-regulated by doxycycline added to the culture media (see M&M section). Over-expression of Dbp2p stimulated TBSV repRNA accumulation by 36% (Fig. 8B, lanes 13–16), while that of AtRH20 by 47% (Fig. 8B, lanes 21–24). The over-expression of Dbp2p in yeast with down-regulated Ded1p level did not increase p33 and p92^{pol} expression level (Fig. 8B), suggesting that Dbp2p could directly complement the defect in TBSV repRNA replication, not via increasing translation of the viral replication proteins. Similar observation can be made for AtRH20 helicase, whose over-expression did not change the amount of p33 and p92^{pol} expressed in yeast with down-regulated Ded1p level (Fig. 8B). Overall, these data suggest that over-expression of Dbp2p or AtRH20 helicases increase TBSV replication and Dbp2p or AtRH20 can complement the replication defect seen in yeast with reduced Ded1p level.

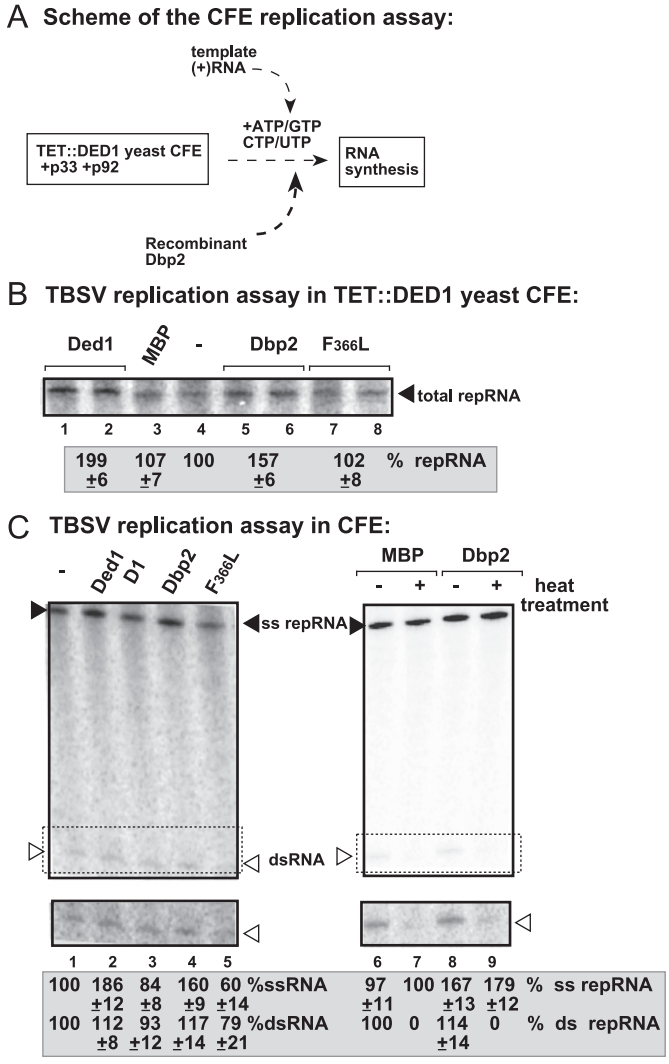

Fig. 5. A stimulatory role for Dbp2p helicase in plus-strand synthesis based on cell-free TBSV replication assay. (A) Scheme of the CFE-based TBSV replication assay with added purified recombinant p33 and p92^{pol} replication proteins of TBSV and *in vitro* transcribed TBSV DI-72 (+)repRNA. The whole cell extract (CFE) was prepared from Ded1p-depleted yeast strain to decrease the amount of endogenous DEAD-box helicase present in the tombusvirus replicase complex. The assay also contained purified recombinant helicase proteins. (B) Denaturing PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assay in the presence of Dbp2p (0.3 μg) or dbp2-F366L ATPase inactive mutant or Ded1p (0.3 μg) as a control. Each experiment was repeated three times. (C) Top panels: Nondenaturing PAGE analysis of TBSV replication assay based on CFE. Detection of single- and double-stranded RNA products produced in the cell-free TBSV replication assay. Note that the dsRNA product represents the annealed (−)RNA and the (+)RNA, while the ssRNA products represents the newly made (+)RNA products. Samples in lanes 7 and 9 were heat-treated to demonstrate the dsRNA nature of the fast migrating product, while the other samples were not heat-treated (thus both ssRNA and dsRNA products are present). Note that the heat-treatment denatures dsRNA, leading to ^{32}P-labeled ssRNA(+) and ssRNA(−) products that co-migrate in this gel with "ss repRNA". Lower panel: A higher contrast image of portion of the top panels to aid the visualization of the dsRNA products. The % of dsRNA and ssRNA in the samples are shown. Each experiment was repeated three times.

Discussion

RNA viruses co-opt numerous host RBPs to assist their replication. The host RBPs have been shown to affect either (−)- or (+)-strand RNA synthesis, stabilize the viral RNA and be involved in recruitment of the viral RNA into replication (Li and Nagy, 2011; Nagy and Pogany, 2011; Ogram and Flanegan, 2011). Tombusviruses with small genomes rely on several RBPs, such as eEF1A, eEF1Bγ, GAPDH and Ded1p, which regulate viral RNA synthesis by the VRC (Nagy and Pogany, 2010; Pogany et al., 2008; Wang and Nagy, 2008; Wang et al., 2009b, 2009c)). Among the RBPs, RNA helicases, which are involved in unwinding RNA structures and remodeling RNA-protein complexes, are likely involved in viral RNA replication. Unlike large RNA viruses, tombusviruses and other small RNA viruses do not code for their own helicases (Koonin and Dolja, 1993; Zuniga et al., 2009). These viruses likely recruit host helicases in order to facilitate viral replication. Accordingly, previous genome-wide screens and global proteomics approaches have identified several yeast helicases that are likely involved in TBSV replication (Li et al., 2008, 2009; Panavas et al., 2005). But how do these helicases assist tombusvirus replication? The best-characterized member of the helicase family involved in tombusvirus replication is Ded1p DEAD-box helicase, which promotes (+)-strand synthesis (Kovalev et al., 2012). Ded1p binds to the 3′-end of the minus-strand RNA, rendering it easily accessible to p92^{pol} for repeated initiation to produce abundant (+)-strand RNA progeny. The additional host RNA helicases identified in previous screens for TBSV replication might have a similar, redundant role with Ded1p, or they could be involved in other viral replication steps.

It seems that the mostly cytosolic Dbp2p DEAD-box helicase, which is normally involved in nonsense-mediated mRNA decay and ribosome biogenesis (Bond et al., 2001), is recruited by TBSV to promote viral replication. The supporting evidence for the role of Dbp2p in TBSV replication is convincing and includes: (i) recombinant Dbp2p binds to both (+) and (−) TBSV repRNA *in vitro* (Fig. 1); (ii) Dbp2p has been co-purified with TBSV repRNA from yeast cells (Li et al., 2009); (iii) addition of purified Dbp2p to an *in vitro* TBSV replication assay based on purified tombusvirus replicase led to increased (+)-strand production (Fig. 3); (iv) the purified Dbp2p also promoted (+)-strand synthesis in a CFE-based TBSV replication assay (Fig. 5); (v) over-expression of Dbp2p increased TBSV repRNA replication in yeast (Fig. 8) (Li et al., 2009); and (vi) an ATPase inactive mutant of Dbp2p inhibited TBSV replication *in vitro* and *in vivo*.

The role of Dbp2p seems to be overlapping with Ded1p, since both helicases bind to the 3′-end of the (−)-strand; they promote TBSV (+)-strand synthesis based on *in vitro* replication assays with purified viral replicase or CFE-based assay; and they require functional ATPase activity. Also, Dbp2p can complement Ded1p activity in a CFE-based assay utilizing CFE prep with low level of Ded1p (Figs. 5 and 6) or in yeast with down-regulated Ded1p (Fig. 8B). Thus, both these RNA helicases might play redundant roles in TBSV replication. On the other hand, Ded1p also affects the translation of viral replication proteins (Jiang et al., 2006;

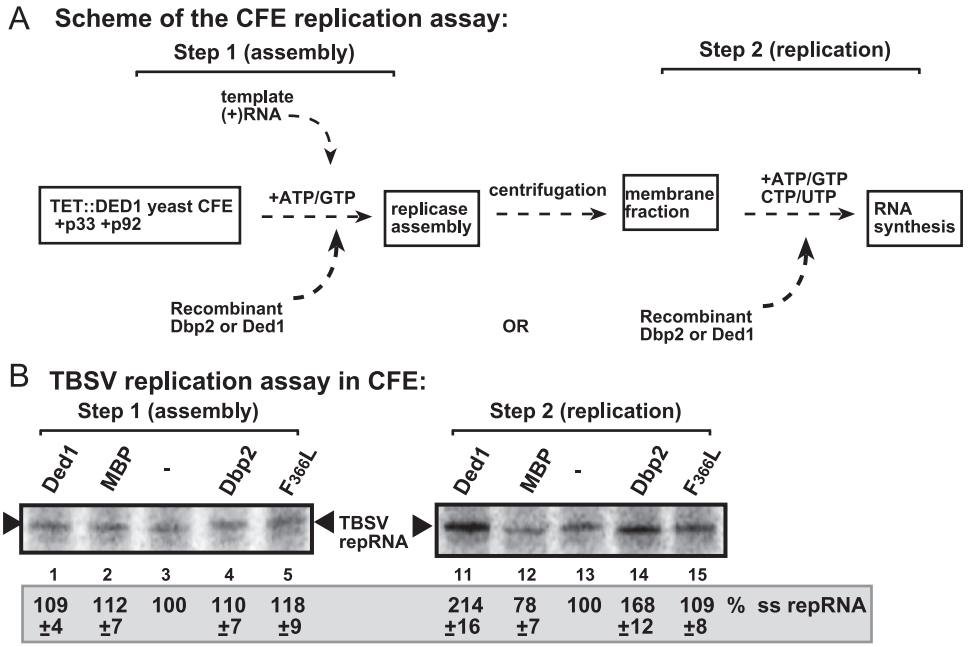

Fig. 6. Lack of a role for Dbp2p helicase in the assembly of the TBSV VRC based on step-wise cell-free TBSV replication assay. (A) Scheme of the CFE-based TBSV VRC assembly and replication assays. Purified recombinant p33 and p92^{pol} replication proteins of TBSV and *in vitro* transcribed TBSV DI-72 (+)repRNA were added in step 1 to the CFE prepared from Ded1p-depleted yeast strain to decrease the amount of endogenous DEAD-box helicase present in the tombusvirus replicase complex. The assay was performed with or without the purified recombinant Dbp2p (0.3 μg) or Ded1p (0.3 μg) during step 1. Note that the assay was performed in the presence of ATP/GTP to facilitate TBSV VRC assembly, but inhibit the RNA synthesis in step 1. After finishing step 1, we centrifuged the samples to collect the membrane fraction of the CFE, followed by washing the membranes, and performing step 2 in the presence of ATP/CTP/GTP and ^{32}P-UTP to allow TBSV RNA replication. The samples presented in right panel were obtained by adding Dbp2p and Ded1p only at the beginning of step 2. (B) Denaturing PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays in the presence of Dbp2p and Ded1p. See further details in Fig. 4. Each experiment was repeated at least three times and the data were used to calculate standard deviation.

Serviene et al., 2006), while over-expression of Dbp2p did not seem to affect the level of the replication proteins (Fig. 8B). Thus, the proposed redundant function of Ded1p and Dbp2p is manifested only during TBSV replication, but not translation. Unfortunately, we could not do the reciprocal set of experiments, since we could not obtain yeast strains with either deletion of *DBP2* gene or down-regulation (these strains are also missing from yeast libraries) possibly due to the essential function of Dbp2p for yeast growth.

RNA helicases form large protein families in yeast (20 proteins) or *Arabidopsis* (more than 50 proteins), which might perform similar functions to Dbp2p or Ded1p during TBSV replication. Based on our *in vitro* data and over-expression experiments in yeast, it seems that AtRH20 plant helicase protein, which is very similar to Dbp2p (Fig. 2) and Ded1p, can likely perform similar function in plant infections with tombusviruses. Due to the large number of RNA helicases present in plants, there is a need for further experiments to define if additional host helicases might also be involved in TBSV replication.

Role of Dbp2p and AtRH20 helicases in stimulation of TBSV RNA synthesis

The *in vitro* data with the solubilized and purified tombusvirus replicase and the CFE-based assay containing the membrane-bound VRC demonstrated a role for Dbp2p and AtRH20 helicases in stimulation of TBSV (+)-strand synthesis. Interestingly, the ATPase function of Dbp2p is required for the stimulatory effect on (+)-strand synthesis, suggesting that Dbp2p functions in unwinding the secondary structure of the (−)RNA template. Indeed, using a partial DNA/RNA hybrid that "masks" the plus-strand initiation promoter, we show that Dbp2p can facilitate initiation by the tombusvirus replicase, likely due to destabilizing the partial DNA/RNA hybrid prior to initiation of RNA synthesis.

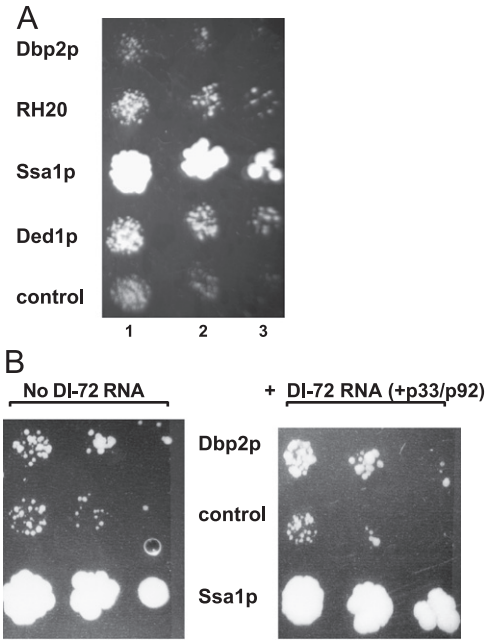

Fig. 7. Interaction between Dbp2p and the tombusvirus p33 replication protein is facilitated by the viral RNA. (A) Split-ubiquitin assay was used to test binding between p33 and the shown full-length host proteins. The bait p33 was co-expressed with the prey proteins in yeast. Ssa1p (HSP70 chaperone), and the empty prey vector (NubG) were used as positive and negative controls, respectively. The image shows 10-fold serial dilutions of yeast cultures. Note the lack of interaction between Dbp2p and p33 based on comparison with the negative control. (B) A modified split-ubiquitin assay shows that p33 could interact with Dbp2p in yeast replicating the TBSV repRNA. The left panel shows the split-ubiquitin assay in the absence of TBSV replication, while the right panel demonstrates interaction between Dbp2p and p33 in the presence of TBSV repRNA replication. All yeasts shown in the right panel expresses p33, p92 and the DI-72 repRNA.

Based on our data, we propose that Dbp2p and AtRH20 directly affect TBSV RNA synthesis via affecting the structure of the RNA templates (Fig. 9). This function of Dbp2p overlaps with that of Ded1p DEAD-box helicase, suggesting that TBSV could recruit more than one DEAD-box helicases to facilitate (+)-strand synthesis. Similar situation likely exists in plants, in which many members of the DEAD-box helicases with similar sequence to Dbp2p can be found. The list includes AtRH20 with the highest similarity (Fig. 2), followed by AtRH30, AtRH14, AtDRH1, AtRH46 and AtRH40 with high sequence similarity (not shown).

We propose a model in which these DEAD-box helicase proteins are needed for robust TBSV (+)RNA synthesis that leads to excess amount of progeny (+)RNA (Fig. 9). The major function of these helicases could be the remodeling of the viral RNA bound by the viral RdRp or host proteins prior or during RNA synthesis. For example, the DEAD-box helicases might unwind either local secondary structure in (−)repRNA or dsRNA region, which could favor binding of the (−)RNA by the viral $p92^{pol}$ replication protein or by GAPDH host protein involved in (+)-strand synthesis (Huang and Nagy, 2011; Kovalev et al., 2012; Wang and Nagy, 2008). Therefore, the emerging picture is that TBSV utilizes several host DEAD-box helicases for promoting (+)RNA synthesis. The redundant use of helicases could be beneficial for TBSV by allowing efficient recruitment of these host factors to allow asymmetric RNA synthesis, leading to excess amount of progeny (+)RNA.

Similar RNA helicases to Dbp2p and AtRH20 might also be involved in other RNA virus infections. For example, the human p68 DEAD-box helicase, the homolog of the yeast Dbp2p, has been found to interact with the RdRp protein of human hepatitis C virus (HCV) and p68 is relocalized to the cytoplasm, the site of replication, in HCV-infected cells (Goh et al., 2004; Lee et al., 2006; Lim et al., 2006). Thus, p68 might play similar function during HCV replication to Dbp2p and Ded1p helicases during TBSV replication. Also, a p68-related DEAD-box helicase, called p72 from human cells is known to interact with zinc-finger antiviral protein (ZAP). The p72-ZAP interaction is required for inhibition of many human viruses by binding of ZAP to viral RNAs or viral mRNAs, followed by recruitment of the RNA exosome to degrade the viral RNA targets (Chen et al., 2008).

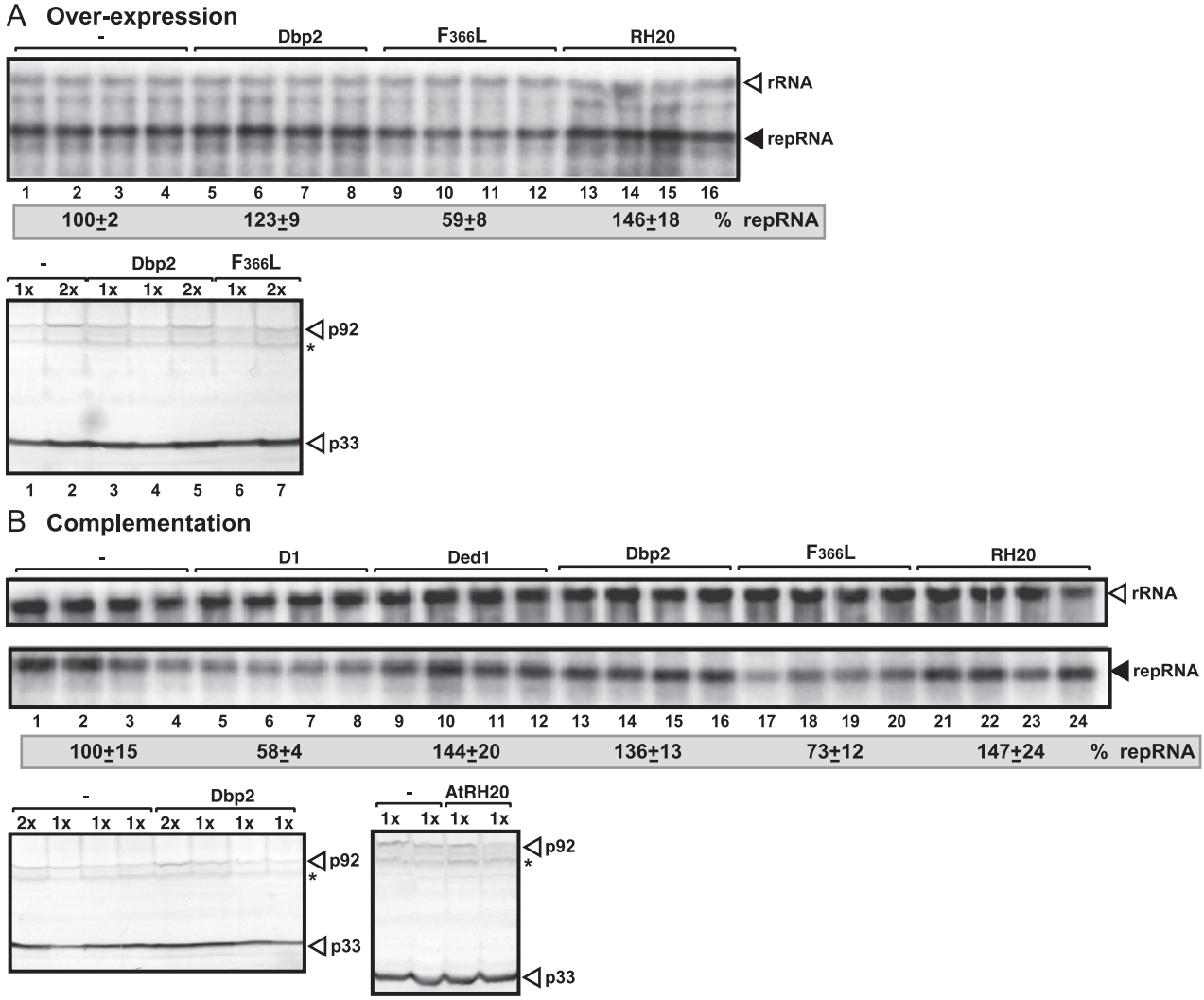

Fig. 8. Dbp2p and AtRH20 DEAD-box helicase complements Ded1p helicase during TBSV replication. (A) Top panel: Northern blot analysis was used to detect DI-72(+) repRNA accumulation in BY4741 wt yeast strain expressing Dbp2p, dbp2-F366L or AtRH20 from the *GAL1* promoter. Note that samples were obtained after 24 h of repRNA replication. Bottom panel: Western blot analysis of the accumulation level of His_6-tagged p33, and His_6-tagged p92 using anti-His_6 antibody. The asterisk mark (*) indicates p33 dimer-like proteins. (B) Top panel: Northern blot analysis was used to detect DI-72(+) repRNA accumulation in TET::Ded1 yeast strain (grown in the presence of doxycycline) expressing Dbp2p, dbp2-F366L, Ded1p, D1 (ATPase inactive mutant of Ded1p) or AtRH20 from the *GAL1* promoter. Note that samples were obtained after 18 h of repRNA replication. See further details in Panel A. Bottom panel: Western blot analysis of the accumulation level of His_6-tagged p33, and His_6-tagged p92 using anti-His_6 antibody.

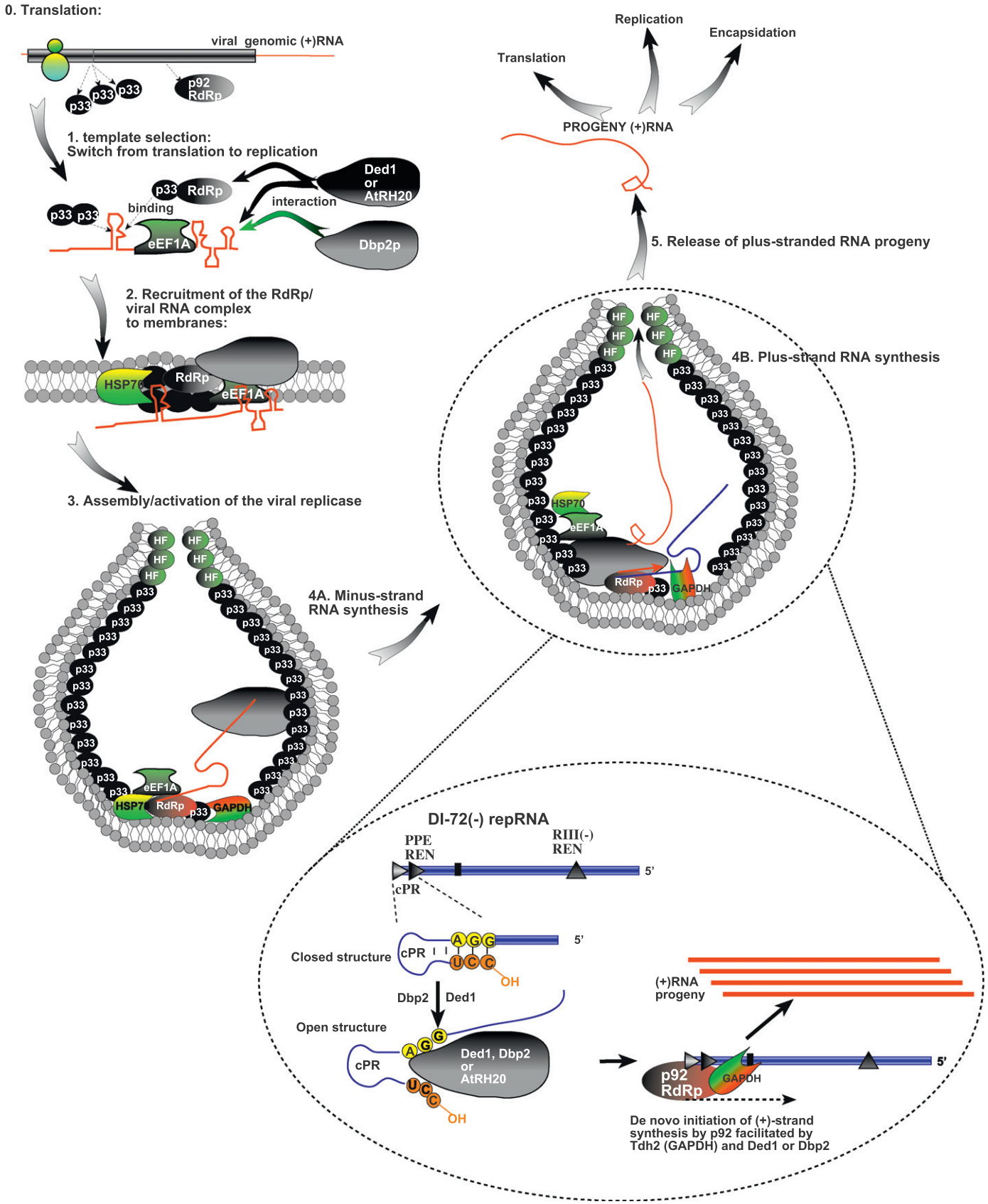

Fig. 9. A model describing the functions of Dbp2p and AtRH20 during tombusvirus replication. Dbp2p and AtRH20 might be recruited by TBSV via binding to the viral (+)RNA in the cytosol. Then, Dbp2p and AtRH20 are proposed to facilitate the use of (−)repRNA as template for (+)strand synthesis within the VRC. The helicase function of Dbp2p and AtRH20 might help the opening of a "closed" 3′ end, which could form secondary structure including the promoter (cPR) in the (−)repRNA or be part of a dsRNA structure (see encircled, enlarged portion of the image for details). The resulting open ssRNA structure would facilitate (+)RNA synthesis by the tombusvirus p92 RdRp protein as shown. We propose that Ded1p, Dbp2p and AtRH20 play similar roles in this process.

Another similar host protein, RHA (also called DHX9) ATP-dependent DEXD/H box helicase modulates the replication of several RNA viruses, such as HCV, foot-and-mouth disease virus, influenza A virus and HIV-1 (Bolinger et al., 2010; Isken et al., 2007; Lawrence and Rieder, 2009; Lin et al., 2012; Xing et al., 2011). RHA is associated with the Gag protein of HIV and it is incorporated into HIV-1 particles and promotes the annealing between the host tRNA primer and the HIV RNA (Xing et al., 2011).

Additional host DEAD-box or other RNA helicases have been shown to affect translation of viral proteins (Bolinger et al., 2010; Noueiry et al., 2000; Watanabe et al., 2009) or viral RNA replication (Goh et al., 2004; Huang et al., 2010; Lawrence and Rieder, 2009; Morohashi et al., 2011). Host RNA helicases also modify reverse transcription (Wang et al., 2009a); the activity of antiviral proteins (Sumpter et al., 2005; Ye et al., 2010), and virus-mediated regulation of host gene transcription (Jong et al., 2010). All these findings suggest that DEAD-box helicases are important for many RNA viruses.

Materials and methods

Yeast strains and expression plasmids

S. cerevisiae strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and TET::DED1 yeast strain (yTHC library, *MAT***a** *his3Δ1 leu2Δ0 met15Δ0* URA3::CMV-tTA) were obtained from Open Biosystems (Huntsville, AL, USA). The plasmid pESC-HIS-Gal-His33/Gal-DI-72 co-expressing *Cucumber necrosis virus* (CNV) His_6-tagged p33 and the TBSV DI-72 repRNA was described earlier (Pathak et al., 2008).

Yeast plasmids (pESC-Leu-Galp92-GalHP) co-expressing CNV His_6-tagged p92 and one of the helicase proteins (HP) were prepared as follows. The cDNA of His_6-P92 sequence was PCR-amplified from pGAD-His92 plasmid (Panaviene et al., 2004) using primers 1402 (GCGGCAGATCTTACCATGGGGGGTTCTCA) and 952 (CCCGCTCGAGTCATGCTACGGCGGAGTCAAGGA). The obtained PCR product was inserted into pESC-Leu plasmid (Invitrogen) under *GAL1* promoter using BglII and XhoI restriction sites.

The various cDNAs for the HP sequences were PCR-amplified as follows: For wt and D1 mutant of *DED1,* using primers 4468 (CCAGTTAATTAAATGGCTGAACTGAGCGAACAAG) and 4469 (CCAGACTAGTTCACCACCAAGAAGAGTTG); for *DBP2* and its mutant, 4470 (CCAGTTAATTAAATGACTTACGGTGGTAGAG) and 4471 (CCAGACTAGTTCAATAGTTTGAACGACCTC); for *AtRH20*, 4472 (CCAGTTAATTAAATGAGTCGCTACGATAGCCG) and 4473 (CCAGACTAGTTCAGCTCCACCCTCTTCTGCTC) and pMal-Ded1, pMal-D1, pMAL-RH20 (Kovalev et al., 2012), pMAL-Dbp2 and pMAL-Dbp2mut as templates, respectively. The obtained PCR products were digested with PacI/SpeI and inserted into pESC-Leu-p92 plasmid (described above) under *GAL10*, which was digested with the same pair of enzymes.

Plasmids pGAD/Cup/FHV/proteinA/C-term/HA/FLAG for expression of recombinant FHV protein A in yeast and pESC-His-GAL1: FHVRNA1frameshift were described earlier (Kovalev et al., 2012).

Gel mobility shift assay (EMSA)

EMSA was performed as published previously (Pogany et al., 2005), except that the binding assay was done in the presence of 20 mM HEPES (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA 5% glycerol, 6 U of RNasin and 0.1 μg tRNA in a 10 μl reaction volume. We used 0.1 pmol of ^{32}P-labeled RNA probes, 0.6 μg of recombinant proteins and 0.3 μg of unlabled RNA in competition assay. Reaction mixtures were incubated at room temperature for 15 min and loaded on 5% nondenaturing polyacrylamide gel as published previously (Rajendran and Nagy, 2003).

Expression and purification of recombinant proteins from E. coli

Recombinant proteins Dbp2p and dbp2-F366L mutant were produced in *E. coli* as maltose binding protein (MBP) fusions (Rajendran et al., 2002). The expression plasmid pMal-DBP2 was described earlier (Li et al., 2009). The expression plasmid pMal-DBP2mut (dbp2-F366L mutant) was prepared by using PCR with primers 4312 (CCAGGGATCCATGACTTACGGTGGTAGAG) and 4313 (CCAGCTGCAGTCAATAGTTTGAACGACCTC) and dbp2-F366L plasmid as a template (Banroques et al., 2008). The PCR product was then inserted between BamHI and PstI sites in pMalc-2X (NE Biolabs).

Expression and purification of the recombinant MBP-tagged host proteins and the MBP-tagged TBSV p33 and p92 replication proteins from *E. coli* was carried out as described earlier (Rajendran and Nagy, 2003; Rajendran et al., 2002). Briefly, the expression plasmids were transformed into *E. coli* strain BL21(DE3) CodonPlus. Isopropyl β-D-thiogalactopyranoside (IPTG) was used for inducing protein expression for 8 h at 16 °C in the case of p33 and p92 and at 23 °C in the case of host proteins. The cells were collected by centrifugation (5000 rpm for 5 min) and after that cells were suspended and sonicated in MBP low-salt column buffer (30 mM HEPES-KOH pH 7.4, 25 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol). The lysate was then centrifuged at 14,000 rpm for 5 min to remove cell debris, followed by incubation of supernatant with amylose resin (NEB) for 15 min at 4 °C with mixing. The resin was washed 2 times with the column buffer and the bound proteins were eluted with column buffer containing 0.18% (V/W) maltose. Eluted proteins were aliquoted and stored at −80 °C. Proteins used for the replication assays were at least 95% pure, as determined by SDS-PAGE (not shown).

Preparation of RNA probes and RNA competitors used for RNA:protein interactions

The ^{32}P-labeled or unlabeled TBSV repRNA-derived sequences [*i.e.*, the four separate regions (RI-IV) and full-length DI-72 (+) and (−)RNAs] were generated using T7 transcription as described (Rajendran and Nagy, 2003; Rajendran et al., 2002). Transcripts for CFE-based replication assays or replicase assays were purified as described earlier (Rajendran and Nagy, 2003; Rajendran et al., 2002). The quantification of RNAs transcripts was done using a UV spectrophotometer (Beckman). Full-length FHV-derived DI-634 RNA was generated as described (Kovalev et al., 2012).

Strand separation assay

Strand separation assay was performed as described (Stork et al., 2011). Briefly, oligo 20 (5′–GGAAATTCTCCAGGATTTCTC) was ^{32}P-labeled at the 5′-end with γATP (0.025 mCi) using T4 polynucleotide kinase (Fermentas). The annealing of 1 pmol of oligo 20 and 1 pmol of DI-72(−)RNA was done in an STE buffer (10 mM TRIS, pH 8.0, 1 mM EDTA, and 100 mM NaCl) by slowly cooling down the samples from 94 to 25 °C in 30 min. Different amounts of MBP, MBP-DBP2, or MBP-DBP2mut (dbp2-F366L) proteins were added separately to the ssDNA/ssRNA duplex in the RdRp buffer, followed by incubation at room temperature for 15 min. The samples were then analyzed by electrophoresis on non-denaturing 5% acrylamide gels, followed by phosphoimaging (Stork et al., 2011).

In vitro TBSV replication assay in cell-free yeast extract

CFE preparation using TET::DED1 yeast strain was prepared as described (Kovalev et al., 2012; Pogany and Nagy, 2008). The *in vitro* TBSV replication assays based on CFE were performed in 20 μl total volume containing 2 μl of CFE, 0.25 μg DI-72 (+)repRNA transcript, 200 ng purified MBP-p33, 200 ng purified MBP-p92^{pol} (both recombinant proteins were purified from *E. coli*) (Kovalev et al., 2012; Pogany et al., 2008), 30 mM HEPES-KOH, pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate, 0.13 M sorbitol, 0.4 μl actinomycin D (5 mg/ml), 2 μl of 150 mM creatine phosphate, 0.2 μl of 10 mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 0.2 μl of 1 M dithiothreitol (DTT), 2 μl of rNTP mixture (10 mM ATP, CTP, and GTP and 0.25 mM UTP) and 0.1 μl of [^{32}P]UTP. Purified recombinant host proteins were added separately in different amounts as described in the figure legends. The assays were performed at 25 °C for 3 h. The RNA products synthesized in replication reaction were ^{32}P-labeled and were separated by electrophoresis in 0.5× Tris–borate-EDTA (TBE) buffer in a 5% polyacrylamide gel (PAGE) containing 8 M urea. For detection of the double-stranded RNA (dsRNA) in *in vitro* replication reaction, the obtained RNA samples were divided into two halves: one half was loaded onto the gel without heat treatment in the presence of 25% formamide, while the other half was heat-denatured at 85 °C for 5 min in the presence of 50% formamide (Li et al., 2010).

Fractionation of the whole cell extract, CFE, was described (Pogany and Nagy, 2008). The CFE was centrifuged at 14,000 rpm at 4 °C for 10 min to separate the "soluble" (supernatant) and "membrane" (pellet) fraction. The membrane fraction was re-suspended and washed with buffer A (30 mM HEPES-KOH pH 7.4, 150 mM potassium acetate, and 5 mM magnesium acetate) followed by centrifugation at 14,000 rpm at 4 °C for 10 min and re-suspension of the pellet in buffer A. *In vitro* replication assays in the fractions were performed as described (Pogany et al., 2008).

Purification of tombusvirus and FHV replicases from yeast and in vitro replicase assays

TET::DED1 yeast strain was transformed with plasmids pESC-DI72-HisFlag33 and pGAD-HisFlag92 (Kovalev et al., 2012; Pathak et al., 2008). The His_6-Flag double-tagged p92 was expressed from the *ADH1* promoter and HF-p33 and DI-72 repRNA were under the *GAL1* and *GAL10* promoters, respectively (Kovalev et al., 2012). Transformed yeast cells were pre-grown in SC-ULH$^-$ media containing 2% glucose at 29 °C. After centrifugation at 2000 rpm for 3 min and washing pellet with selective media containing 2% galactose, yeast cells were grown in SC-ULH$^-$ media containing 2% galactose and 1 mg/ml Doxycycline at 23 °C for 24 h. The tombusvirus replicase purification was done as described previously (Panaviene et al., 2004; Serva and Nagy, 2006). Briefly, 200 mg of yeast cells were re-suspended and homogenized by glass beads using FastPrep Homogenizer (MP Biomedicals) in TG buffer [50 mM Tris–HCl [pH 7.5], 10% glycerol, 15 mM $mgCl_2$, 10 mM KCl, 0.5 M NaCl, and 1% [V/V] yeast protease inhibitor cocktail (Ypic)]. After centrifugation at 38,000 *g* for 15 min, pellet containing the membrane-bound viral replicase complex was solubilized with 1 ml TG buffer containing 1% Triton X-100, 1% [V/V] Ypic as described (Panaviene et al., 2004; Serva and Nagy, 2006). After affinity purification of Flag-tagged replicase proteins on anti-FLAG M2-agarose affinity resin (Sigma), the resin-bound replicase complex was eluted in 100 μl elution buffer [50 mM Tris–HCl [pH 7.5], 10% glycerol, 15 mM $mgCl_2$, 10 mM KCl, 50 mM NaCl, 1% Triton X-100, and 0.15 mg/ml Flag peptide (Sigma)].

To purify FHV replicase, BY4741 yeast strain was transformed with plasmid pGAD/Cup/FHV/proteinA/C-term/HA/FLAG and pESC-His-GAL1::FHVRNA1framshift (Kovalev et al., 2012). Transformed yeast cells were selected on SC-LH$^-$ plates. Yeast cells were pre-grown overnight in selective media containing 2% glucose at 29 °C, and after centrifugation at 2000 rpm for 3 min and washing pellet with selective media containing 2% galactose, yeast cells were grown for 36 h at 29 °C in SC-LH$^-$ media containing 2% galactose and 50 μM $CuSO_4$ to induce FHV RNA replication. FHV replicase was purified using the same procedure as for tombusvirus, except for using different buffers (Kovalev et al., 2012): cell lysis buffer consisted of 50 mM Tris–HCl [pH 8.0], 0.4 M sorbitol, 5 mM $mgCl_2$, 50 mM KCl, 0.03% β-mercaptoethanol and 1% [V/V] yeast protease inhibitor cocktail (Ypic)] and solubilization (washing) and elution buffers contained 50 mM Tris–HCl [pH 8.0], 0.4 M sorbitol, 5 mM $mgCl_2$, 50 mM KCl, 0.5 M NaCl, 0.03% β-mercaptoethanol, 1% Triton X-100 and 1% [V/V] yeast protease inhibitor cocktail (Ypic)]. Elution buffer also contained 0.15 mg/ml Flag peptide (Sigma)]. DI-72(−) or (+) RNA template transcribed *in vitro* by T7 transcription was used in *in vitro* FHV replicase activity assay (Kovalev et al., 2012). The effect of host helicase proteins on DNA/RNA duplex containing 21 bp double-stranded region was measured in the replicase assay with DI-72(−) RNA annealed with oligodeoxynucleotide 20 or DI-634 RNA annealed with oligodeoxynucleotide 3519 at the 3′-ends (Kovalev et al., 2012).

Analysis of protein–protein interactions using the split-ubiquitin assay

The replicase protein:helicase interaction assay was based on the split-ubiquitin assay (Dualmembrane kit3, Dualsystems). The bait construct, expressing the CNV p33 (pGAD-BT2-N-His33) replication protein has been published earlier (Li et al., 2008). The prey DBP2 constructs were made by PCR amplification of gene sequence from pMAL-DBP2 plasmid using gene specific primers: 4312 (CCAGGGATCCATGACTTACGGTGGTAGAG) and 4603 (CCAGCCATGGATAGTTTGAACGACCTC), followed by digestion with *BamH1* and *Nco1*. The digested PCR products were fused to NubG at the 5′- terminus (NubG-x) by cloning into pPRN-N-RE plasmid (Li et al., 2008), using the same enzymes. Yeast strain NMY51 was co-transformed with pGAD-BT2-N-His33 and either pPR-N-DBP2 or pPR-N-RE (as a negative control) and plated on Trp$^-$/Leu$^-$ synthetic minimal medium plates. Obtained colonies were picked with a loop, re-suspended in water, and streaked on TLHA$^-$ (Trp$^-$/Leu$^-$/His$^-$/Ade$^-$) plates to test for p33-helicase protein interactions as described (Li et al., 2008).

Analysis of protein–protein interactions using the split-ubiquitin assay in the presence of DI72 RNA

The plasmid pUraGBK-His33/DI-72 was created by replacing the *HIS3* marker gene to *URA3* in plasmid pGBK-His33/DI-72 (Jiang et al., 2006). To do so, the *URA3* gene was PCR-amplified using plasmid pYC2/CT (Invitrogen) with primers 1627 (cgcgTCCGGAGAAAAG-GACCCATCGATAAGCTAGCT) and 1628 (CGCGAAGzCTTCTCTTCC-TTTTTCAATGGGTAATAACT). A DNA portion containing the F1 ori was amplified from plasmid pGBK-His33/DI-72 with primers 1629 (cgcgAAGCTTGCACTTGCCGATCTATGCGGTGTGA) and 1545 (CCGGT-CGAGCTCATCGCCCTTCCCAACAGTTGCGCA). The two PCR products were digested with *Hind*III, ligated and re-amplified by PCR using primers 1627 and 1545. The resulting PCR product was digested with *Bsp*EI and *Sac*I and inserted into *Bsp*EI/*Sac*I-digested pGBK-His33/DI-72 to create pUraGBK-His33/DI-72. To replace the *GAL*1 promoter driving DI-72 expression for a *CYC*1 promoter, the *CYC*1 sequence was amplified from plasmid pPR-N-RE (Li et al., 2008) with primers 4393 (CGGCAGATCTTTAGTGTGTGTATTTGTGTTTGC) and 4394 (CGGCAGATCTTTAGTGTGTGTATTTGTGTTTGC). The DI-72

sequence including the ribozyme sequence at the 3′ end was PCR-amplified using pYC/DI-72 (Panavas and Nagy, 2003) with primers 1803 (GGCGAGATCTGGAAATTCTCCAGGATTTCTC) and 1069 (CCGG-TCGAGCTCTACCAGGTAATATACCACAACGTGTGT). The two PCR products were digested with *Bgl*II, then ligated and re-amplified with primers 1069 and 4393. The resulting product was digested with *Sac*I and *Eco*RI and inserted into *Sac*I/*Eco*RI-digested pUraGBK-His33/DI-72 generating pUraGBK-His33/CYC1-DI-72. Additionally, the *Sac*I/*Eco*RI-digested pUraGBK-His33/DI-72 plasmid was treated with T4 DNA polymerase to generate blunt ends and then re-ligated to create plasmid pUraGBK-His33.

A cassette containing the *ADH*1 promoter, His_6-tagged CNV p92 and the *ADH*1 terminator was PCR-amplified with primers 3655 (CTAATGAATCCATTTGTTTGTTAATAGTTTGAATGTTTTTTTTGTTGTT-TCCGGGTGTAC) and 2756 (GCCGAGATCTGAGCGACCTCATGCTA-TAC) using pGAD-His92 as template (Panaviene et al., 2004). The resulting product was digested with *Bgl*II and ligated to *Bgl*II-digested pYM-14 (EUROSCARF) (Janke et al., 2004), which contains a *KanMX4* gene providing resistance to G418. The resulting cassette containing the ADH-6xHis-p92 and *KanMX4* genes was amplified with primers 3655 and 3653 (CATGGTAGCGCCTGTGCTTCGGT-TACTTCTAAAGAAGTCCAAATCGATGAATTCGAGCTC), which direct chromosomal integration of the PCR product at retrotransposon sites. The resulting PCR product was transformed into yeast strain NMY51 (DUAL SYSTEMS) to generate NMY51/ADH-His92-Kan. The obtained yeast strain NMY51/ADH-His92-Kan was co-transformed with bait construct pGAD-BT2-N-His33, either prey construct pPR-N-DBP2 or pPR-N-RE as a negative control and pUraGBK-His33/CYC1-DI-72 to have full replication cycle (including replicating DI72) in yeast or pUraGBK-His33 as a control with absent DI72 RNA. Colonies were selected on Trp^-/Leu^-/Ura^- synthetic minimal medium plates. Obtained colonies were picked with a loop, re-suspended in water, and streaked on TLUHA$^-$ (Trp^-/Leu^-/Ura^-/His^-/Ade^-) plates to test for p33-helicase protein interactions in the presence or in absence of replicating DI72.

Complementation studies in yeast

In order to study the effect of over-expression of Dbp2p and AtRH20 on TBSV repRNA replication in yeast, we transformed *S. cerevisiae* parental strain (BY4741) or TET::DED1 strain from the yTHC library with two plasmids: pESC-HIS-p33-DI72 and pESC-LEU-p92-DBP2 (or pESC-LEU-p92-DBP2mut, pESC-LEU-p92-DED1, pESC-LEU-p92-D1, pESC-LEU-p92-RH20). Transformed yeast cells were selected on SC-LH$^-$ plates (BY4741) or SC-ULH$^-$ plates (in case of TET::DED1 yeast), then, they were pre-grown in selective media containing 2% glucose for 36 h at 29 °C. We added 10 mg/ml doxycycline to yeast culture 12 h prior to the glucose–galactose media switch to downregulate Ded1p level. After centrifugation at 2000 rpm for 3 min, we washed the pellet with selective media containing 2% galactose. Yeast cells were grown in the appropriate selective media containing 2% galactose for 18 to 24 h at 29 °C. In the case of TET::DED1 yeast strain, the SC-ULH$^-$ media contained 2% galactose and 2% raffinose and 1 mg/ml doxycycline. Total RNA extraction from yeast cells and Northern blotting and Western blotting were done as previously described (Panaviene et al., 2004).

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